ULTRASOUND CONTRAST AGENT DISPERSIONS COMPRISING GAS AND DESTABILISING AGENT

5   This invention relates to ultrasound imaging, more
       particularly to novel contrast agent preparations and
       their use in ultrasound imaging, for example in
       visualising tissue perfusion.
           It is well known that contrast agents comprising
10   dispersions of microbubbles of gases are particularly
       efficient backscatterers of ultrasound by virtue of the
       low density and ease of compressibility of the
       microbubbles. Such microbubble dispersions, if
       appropriately stabilised, may permit highly effective
15   ultrasound visualisation of, for example, the vascular
       system and tissue microvasculature, often at
       advantageously low doses.
           The use of ultrasonography to measure blood
       perfusion (i.e. blood flow per unit of tissue mass) is
20   of potential value in, for example, tumour detection,
       tumour tissue typically having different vascularity
       from healthy tissue, and studies of the myocardium, e.g.
       to detect myocardial infarctions. A problem with the
       application of existing ultrasound contrast agents to
25   cardiac perfusion studies is that the information
       content of images obtained is degraded by attenuation
       caused by contrast agent present in the ventricles of
       the heart.
           In our copending International Patent Publication
30   No. WO-A-9817324, the contents of which are incorporated
       herein by reference, we have disclosed that ultrasonic
       visualisation of a subject, in particular of perfusion
       in the myocardium and other tissues, may be achieved
       and/or enhanced by means of gas-containing contrast
35   agent preparations which promote controllable and
       temporary growth of the gas phase in vivo following
       administration. Such contrast agent preparations may be

used to promote controllable and temporary retention of the gas phase, for example in the form of microbubbles, in tissue microvasculature, thereby enhancing the concentration of gas in such tissue and accordingly enhancing its echogenicity, e.g. relative to the blood pool.

Such use of gas as a deposited perfusion tracer differs markedly from existing proposals regarding intravenously administrable microbubble ultrasound contrast agents. Thus it is generally thought necessary to avoid microbubble growth since, if uncontrolled, this may lead to potentially hazardous tissue embolisation. Accordingly it may be necessary to limit the dose administered and/or to use gas mixtures with compositions selected so as to minimise bubble growth *in vivo* by inhibiting inward diffusion of blood gases into the microbubbles (see e.g. WO-A-9503835 and WO-A-9516467).

In accordance with WO-A-9817324, on the other hand, a composition comprising a dispersed gas phase is coadministered with a composition comprising at least one substance which has or is capable of generating a gas or vapour pressure *in vivo* sufficient to promote controllable growth of the said dispersed gas phase through inward diffusion thereto of molecules of gas or vapour derived from said substance. This latter substance is referred to as a "diffusible component", although it will be appreciated that transport mechanisms other than diffusion may additionally or alternatively be involved.

The preparations may advantageously be employed in visualising tissue perfusion in a subject, the increase in size of the dispersed gas being utilised to effect enrichment or temporary retention of gas in the microvasculature of such tissue, thereby enhancing its echogenicity.

The dispersed gas content of such contrast agent

preparations will tend to be temporarily retained in
tissue in concentrations proportional to the regional
rate of tissue perfusion. Accordingly, when using
ultrasound imaging modalities such as conventional or
harmonic B-mode imaging where the display is derived
directly from return signal intensities, images of such
tissue may be interpreted as perfusion maps in which the
displayed signal intensity is a function of local
perfusion. This is in contrast to images obtained using
free-flowing contrast agents, where the regional
concentration of contrast agent and corresponding return
signal intensity depend on the actual blood content
rather than the rate of perfusion of local tissue.

The present invention is based on the finding that
growth of a dispersed gas in order to bring about its
temporary retention in tissue microvasculature may be
effected by administering the gas dispersion in
conjunction with one or more substances serving to
destabilise the dispersion. By avoiding use of a
diffusible component requiring transport through blood
and/or the continuous phase(s) in which the gas
dispersion and diffusible component are contained, as
well as through any encapsulating or other stabilising
membranes which surround them, the invention may allow
greater control of growth of the dispersed gas and may
also involve a higher proportion of gas-containing
structures in the growth process.

Thus according to one aspect of the present
invention there is provided a combined preparation for
simultaneous, separate or sequential use as a contrast
agent in ultrasound imaging, said preparation
comprising:

    i)   an injectable aqueous gas dispersion, and

    ii)   an administrable substance or substances
capable of destabilising said dispersed gas so as at
least transiently to increase the size thereof.

According to a further aspect of the invention

there is provided a method of generating enhanced images
of a human or non-human animal subject which comprises
the steps of:

　　　　i)　　injecting a physiologically acceptable aqueous
medium having gas dispersed therein into the vascular
system of said subject;

　　　　ii)　before, during or after injection of said
aqueous medium administering to said subject a substance
or substances capable of destabilising said dispersed
gas so as at least transiently to increase the size
thereof; and

　　　　iii) generating an ultrasound image of at least a
part of said subject.

　　　　In general any biocompatible gas may be present in
the gas dispersion used in accordance with the
invention, the term "gas" as used herein including any
substances (including mixtures) at least partially, e.g.
substantially or completely, in gaseous or vapour form
at the normal human body temperature of 37°C.
Representative gases thus include air; nitrogen; oxygen;
carbon dioxide; hydrogen; inert gases such as helium,
argon, xenon or krypton; sulphur fluorides such as
sulphur hexafluoride, disulphur decafluoride or
trifluoromethylsulphur pentafluoride; selenium
hexafluoride; optionally halogenated silanes such as
methylsilane or dimethylsilane; low molecular weight
hydrocarbons (e.g. containing up to 7 carbon atoms), for
example alkanes such as methane, ethane, a propane, a
butane or a pentane, cycloalkanes such as cyclopropane,
cyclobutane or cyclopentane, alkenes such as ethylene,
propene, propadiene or a butene, or alkynes such as
acetylene or propyne; ethers such as dimethyl ether;
ketones; esters; halogenated low molecular weight
hydrocarbons (e.g. containing up to 7 carbon atoms); or
mixtures of any of the foregoing. Advantageously at
least some of the halogen atoms in halogenated gases are
fluorine atoms; thus biocompatible halogenated

hydrocarbon gases may, for example, be selected from
bromochlorodifluoromethane, chlorodifluoromethane,
dichlorodifluoromethane, bromotrifluoromethane,
chlorotrifluoromethane, chloropentafluoroethane,
dichlorotetrafluoroethane, chlorotrifluoroethylene,
fluoroethylene, ethylfluoride, 1,1-difluoroethane and
perfluorocarbons. Representative perfluorocarbons
include perfluoroalkanes such as perfluoromethane,
perfluoroethane, perfluoropropanes or perfluorobutanes
(e.g. perfluoro-n-butane, optionally in admixture with
other isomers such as perfluoro-iso-butane);
perfluoroalkenes such as perfluoropropene,
perfluorobutenes (e.g. perfluorobut-2-ene),
perfluorobutadiene, perfluoropentenes (e.g.
perfluoropent-1-ene) or perfluoro-4-methylpent-2-ene;
perfluoroalkynes such as perfluorobut-2-yne; and
perfluorocycloalkanes such as perfluorocyclobutane,
perfluoromethylcyclobutane,
perfluorodimethylcyclobutanes, perfluorotrimethyl-
cyclobutanes, perfluorocyclopentane, perfluoromethyl-
cyclopentane, perfluorodimethylcyclopentanes,
perfluorocyclohexane, perfluoromethylcyclohexane or
perfluorocycloheptane. Other halogenated gases include
methyl chloride, fluorinated (e.g. perfluorinated)
ketones such as perfluoroacetone and fluorinated (e.g.
perfluorinated) ethers such as perfluorodiethyl ether.
The use of perfluorinated gases, for example sulphur
hexafluoride and perfluorocarbons such as
perfluoropropane, perfluorobutanes, perfluoropentanes
and perfluorohexanes, may be particularly advantageous
in view of the recognised high stability in the
bloodstream of microbubbles containing such gases.
Other gases with physicochemical characteristics which
cause them to form highly stable microbubbles in the
bloodstream may likewise be useful.

        The dispersed gas may, for example, be present in
the form of microbubbles at least partially encapsulated

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or otherwise stabilised by gas-stabilising material.
This stabilising material may, for example, comprise an
initially coalescence-resisting surface membrane (for
example gelatin, e.g. as described in WO-A-8002365), a
filmogenic protein (for example an albumin such as human
serum albumin, e.g. as described in US-A-4718433, US-A-
4774958, US-A-4844882, EP-A-0359246, WO-A-9112823, WO-A-
9205806, WO-A-9217213, WO-A-9406477 or WO-A-9501187), a
polymer material (for example a synthetic biodegradable
polymer as described in EP-A-0398935, an elastic
interfacial synthetic polymer membrane as described in
EP-A-0458745, a microparticulate biodegradable
polyaldehyde as described in EP-A-0441468, a
microparticulate N-dicarboxylic acid derivative of a
polyamino acid - polycyclic imide as described in EP-A-
0458079, or a biodegradable polymer as described in WO-
A-9317718 or WO-A-9607434), a non-polymeric and non-
polymerisable wall-forming material (for example as
described in WO-A-9521631), or a surfactant (for example
a polyoxyethylene-polyoxypropylene block copolymer
surfactant such as a Pluronic, a polymer surfactant as
described in WO-A-9506518, or a film-forming surfactant
such as a phospholipid, e.g. as described in WO-A-
9211873, WO-A-9217212, WO-A-9222247, WO-A-9428780, WO-A-
9503835 or WO-A-9729783).

Where phospholipid-containing gas dispersions are
employed in accordance with the invention, e.g. in the
form of phospholipid-stabilised gas microbubbles,
representative examples of useful phospholipids include
lecithins (i.e. phosphatidylcholines), for example
natural lecithins such as egg yolk lecithin or soya bean
lecithin, semisynthetic (e.g. partially or fully
hydrogenated) lecithins and synthetic lecithins such as
dimyristoylphosphatidylcholine,
dipalmitoylphosphatidylcholine or
distearoylphosphatidylcholine; phosphatidic acids;
phosphatidylethanolamines; phosphatidylserines;

phosphatidylglycerols; phosphatidylinositols;
cardiolipins; sphingomyelins; fluorinated analogues of
any of the foregoing; mixtures of any of the foregoing
and mixtures with other lipids such as cholesterol. The

5   use of phospholipids predominantly (e.g. at least 75%)
comprising molecules individually bearing net overall
charge, for example as described in WO-A-9729783, may be
particularly advantageous. Representative negatively
charged phospholipids include naturally occurring (e.g.

10   soya bean or egg yolk derived), semisynthetic (e.g.
partially or fully hydrogenated) and synthetic
phosphatidylserines, phosphatidylglycerols,
phosphatidylinositols, phosphatidic acids and/or
cardiolipins. Representative positively charged

15   phospholipids include esters of phosphatidic acids such
as dipalmitoylphosphatidic acid or distearoyl-
phosphatidic acid with aminoalcohols such as hydroxy-
ethylenediamine.

     The disclosures of all of the above-described
20   documents relating to gas-containing contrast agent
formulations are incorporated herein by reference.

     Gas microbubbles preferably have an initial average
size not exceeding 10 μm (e.g. of 7 μm or less) in order
to permit their free passage through the pulmonary

25   system following administration, e.g. by intravenous
injection. However, larger microbubbles may be employed
where, for example, these contain a mixture of one or
more relatively blood-soluble or otherwise diffusible
gases such as air, oxygen, nitrogen or carbon dioxide

30   with one or more substantially insoluble and non-
diffusible gases such as perfluorocarbons. Outward
diffusion of the soluble/diffusible gas content
following administration will cause such microbubbles
rapidly to shrink to a size which will be determined by

35   the amount of insoluble/non-diffusible gas present and
which may be selected to permit passage of the resulting
microbubbles through the lung capillaries of the

pulmonary system.

The destabilising substance(s) (hereinafter referred to as "the destabiliser") employed to induce at least transient growth of the dispersed gas phase may, for example, operate by mechanisms involving (i) flocculation, aggregation or agglomeration whereby dispersed gas microbubbles assemble together to form clusters; (ii) coalescence or fusion of the dispersed gas moieties whereby two or more such moieties merge to create a larger moiety; or (iii) Ostwald ripening of dispersed gas moieties, where larger microbubbles grow at the expense of smaller ones, the driving force being the higher pressure in small microbubbles compared to large microbubbles as a result of differences in surface tension in accordance with Laplace's law. It will be appreciated that two or more mechanisms may operate simultaneously.

Flocculation, aggregation or agglomeration may be achieved by making the interbubble repulsive forces weaker than the interbubble attractive forces. The size of the resulting clusters may depend on factors such as the initial size and concentration of the microbubbles, the hydrodynamic behaviour of the surrounding blood stream, the ambient temperature and the net attractive force between the microbubbles.

Where the dispersed gas is stabilised by charge interactions, for example by interactions between charged surface groups in stabilising membranes of materials such as charged phospholipids, addition of charged species such as electrolytes will supply counter-ions which will screen the charged surface groups and reduce interbubble repulsive forces. The use of di- and multi-valent counter-ions is preferred; thus, for example, salts, e.g. inorganic salts such as calcium chloride or magnesium chloride may be used to destabilise negatively charged membranes.

Aggregation may also be induced by modifying the

properties of the surrounding water phase, for example
by addition of solvents or of substances which change
the electrical permeability of the medium so as to
destabilise charge-stabilised gas dispersions.

5   Representative examples of such substances include
water-miscible liquids or solids, e.g. aliphatic
alcohols such as ethanol, isopropanol, ethylene glycol,
propylene glycol, glycerol and sorbitol; aliphatic
aldehydes and ketones such as acetaldehyde and acetone;

10   aliphatic esters such as methyl acetate, propyl formate
and ethyl acetate; aliphatic ethers such as methyl
propyl ether and di-isopropyl ether; aliphatic amides
such as N,N-dimethylformamide and N,N-dimethylacetamide;
aliphatic nitriles such as acetonitrile; carbohydrates

15   such as glucose and sucrose; polyethylene glycol;
polypropylene glycol etc. Aliphatic compounds as
described herein typically contain up to 6 carbon atoms.

Other representative destabilisers include
components capable of bridging dispersed gas

20   microbubbles. Examples of such components include
polymers, e.g. polysaccharides such as dextran and
starches; polyaminoacids such as polylysine and proteins
such as gelatin and albumin; polyethers such as
polyethylene glycol, polypropylene glycol and

25   polyoxyethylene-polyoxypropylene block copolymers such
as Pluronics; polyvinyl pyrrolidone etc.

Aggregation may also be induced by adding
components which enter and modify the membrane, thereby
altering the forces between different microbubbles.

30   Examples of such compounds include fatty alcohols, fatty
acids and fatty amines; various surfactants; steroids
such as cholesterol etc.

Where the gas-stabilising material contains acid or
base groups on its surface, destabilisation may be

35   effected by addition of an appropriate acidic or basic
destabiliser in order to change the pH.

Aggregation may be a reversible or irreversible

process, depending on the magnitude of the forces
between the dispersed gas moieties. By optimising these
forces the aggregates may be made loose enough to be
easily disintegrated a short time after their temporary
retention in tissue microvasculature, thus ensuring that
the imaging procedure has a high safety profile. Such
disintegration or redispersion of the aggregates may if
desired be induced or enhanced by subsequent
administration of an appropriate substance. For
instance, a complexing agent such as ethylenediamine
tetraacetic acid may remove or neutralise the effect of
divalent or polyvalent ions used to destabilise charged
microbubbles. A buffer, acid or base may be used to
remove the effect of pH-induced aggregation/
destabilistation. Solvents, e.g. alcohols such as
ethanol, isopropanol, ethylene glycol, propylene glycol
and glycerol; aldehydes and ketones such as acetaldehyde
and acetone; ethers such as methyl propyl ether and di-
isopropyl ether; aliphatic amides such as N,N-
dimethylformamide and N,N-dimethylacetamide; or
aliphatic nitriles such as acetonitrile, may for example
be added to change the bridging behaviour of a polymeric
flocculant by altering the polymer's ability to coil in
the solvent. Such substances therefore re-establish the
repulsive forces between the dispersed gas moieties.

Destabilisers which operate by inducing coalescence
or fusion of the dispersed gas moieties include
substances which modify the properties of gas-
stabilising membrane material so that membrane rupture
between coalescing gas moieties is enhanced.
Representative destabilisers of this type include
surfactants, co-surfactants, solvents and other
substances which penetrate or otherwise modify the
properties of the stabilising membrane. Chemical
modification of the stabilising membrane, e.g. by
addition of electrolytes or by pH change, or chemical
reaction within the stabilising membrane may likewise be

used to induce flocculation.

Growth of dispersed gas moieties by Ostwald
ripening may be initiated by increasing the solubility
of the gas in the surrounding aqueous phase, for example
by adding a solvent or other appropriate component, e.g.
a hydrotrope such as sodium benzoate or acetic acid, or
a surfactant capable of solubilising the gas within
micelles. Representative examples of solvents which may
increase the solubility of the gas in the surrounding
aqueous phase include aliphatic ethers such as ethyl
methyl ether and methyl propyl ether; aliphatic esters
such as methyl acetate, methyl formate and ethyl
formate; aliphatic ketones such as acetone; aliphatic
amides such as N,N-dimethylformamide and N,N-
dimethylacetamide; and aliphatic nitriles such as
acetonitrile. Representative examples of surfactants
which may increase the solubility of the gas in the
surrounding aqueous phase are those forming micelles in
water, e.g. alkyl carboxylates, alkyl sulphonates, alkyl
sulphates, dialkyl sulphosuccinates, alkylpyridinium
salts, alkylammonium salts, alkyl polyethylene glycol
ethers, alkyl polyethylene glycol esters and sorbitol
fatty acid esters; alkyl groups in such surfactants may,
for example, contain 5-30 carbon atoms.

Ostwald ripening kinetics may also be enhanced by
increasing the interfacial tension about the dispersed
gas, for example by modifying the properties of
stabilising membranes, e.g. through solvent addition,
salt addition, pH change or addition of surfactants/ co-
surfactants which provide higher surface tension.

As noted above, the destabiliser may be
administered before, during or after injection of the
gas dispersion; the order of administration and timing
between them when they are separate may be varied to
permit control over the time, rate and thus location of
temporary retention of the dispersed gas.

In many instances the destabiliser will be

administered by injection, e.g. intravenously, although intramuscular or subcutaneous injections may be useful in order to localise the effect of the destabiliser. Volatile destabilisers, e.g. solvents such as ethanol, may, however, also be administered by inhalation; this may be of advantage in avoiding unwanted growth of the dispersed gas in the lung capillaries.

Imaging modalities which may be used in accordance with the invention include two- and three-dimensional imaging techniques such as B-mode imaging (for example using the time-varying amplitude of the signal envelope generated from the fundamental frequency of the emitted ultrasound pulse, from sub-harmonics or higher harmonics thereof or from sum or difference frequencies derived from the emitted pulse and such harmonics, images generated from the fundamental frequency or the second harmonic thereof being preferred), colour Doppler imaging, Doppler amplitude imaging and combinations of these last two techniques with any of the other modalities described above. To reduce the effects of movement, successive images of tissues such as the heart or kidney may be collected with the aid of suitable synchronisation techniques (e.g. gating to the ECG or respiratory movement of the subject). Measurement of changes in resonance frequency or frequency absorption which accompany growth of the dispersed gas may also usefully be made to detect the contrast agent.

It will be appreciated that the dispersed gas content of combined contrast agent preparations according to the invention will, following destabilisation, tend to be temporarily retained in tissue in concentrations proportional to the regional rate of tissue perfusion. Accordingly, when using ultrasound imaging modalities such as conventional or harmonic B-mode imaging where the display is derived directly from return signal intensities, images of such tissue may be interpreted as perfusion maps in which the

displayed signal intensity is a function of local
perfusion. This is in contrast to images obtained using
free-flowing contrast agents, where the regional
concentration of contrast agent and corresponding return
5   signal intensity depend on the actual blood content
rather than the rate of perfusion of local tissue.

In cardiac studies, where perfusion maps are
derived from return signal intensities in accordance
with this embodiment of the invention, it may be
10 advantageous to subject a patient to physical or
pharmacological stress in order to enhance the
distinction, and thus the difference in image
intensities, between normally perfused myocardium and
any myocardial regions supplied by stenotic arteries.
15 As is known from radionuclide cardiac imaging, such
stress induces vasodilatation and increased blood flow
in healthy myocardial tissue, whereas blood flow in
underperfused tissue supplied by a stenotic artery is
substantially unchanged since the capacity for
20 arteriolar vasodilatation is already exhausted by
inherent autoregulation seeking to increase the
restricted blood flow.

The application of stress as physical exercise or
pharmacologically by administration of adrenergic
25 agonists may cause discomfort such as chest pains in
patient groups potentially suffering from heart disease,
and it is therefore preferable to enhance the perfusion
of healthy tissue by administration of a vasodilating
drug, for example selected from adenosine, dipyridamole,
30 nitroglycerine, isosorbide mononitrate, prazosin,
doxazosin, dihydralazine, hydralazine, sodium
nitroprusside, pentoxyphylline, amelodipine, felodipine,
isradipine, nifedipine, nimodipine, verapamil, diltiazem
and nitrous oxide. In the case of adenosine this may
35 lead to in excess of fourfold increases in coronary
blood flow in healthy myocardial tissue, greatly
increasing the uptake and temporary retention of

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contrast agents in accordance with the invention and
thus significantly increasing the difference in return
signal intensities between normal and hypoperfused
myocardial tissue. Because an essentially physical
entrapment process is involved, retention of contrast
agents according to the invention is highly efficient;
this may be compared to the uptake of radionucleide
tracers such as thallium 201 and technetium sestamibi,
which is limited by low contact time between tracer and
tissue and so may require maintenance of vasodilatation
for the whole period of blood pool distribution for the
tracer (e.g. 4-6 minutes for thallium scintigraphy) to
ensure optimum effect. The contrast agents of the
invention, on the other hand, do not suffer such
diffusion or transport limitations, and since their
retention in myocardial tissue may also rapidly be
terminated by the methods described above, the period of
vasodilatation needed to achieve cardiac perfusion
imaging in accordance with this embodiment of the
invention may be very short, for example less than one
minute. This will reduce the duration of any possible
discomfort caused to patients by administration of
vasodilator drugs.

In view of the fact that the required
vasodilatation need only be short lasting, adenosine is
a particularly useful vasodilating drug, being both an
endogenous substance and having a very short-lasting
action as evidenced by a blood pool half-life of only 2
seconds. Vasodilatation will accordingly be most
intense in the heart, since the drug will tend to reach
more distal tissues in less than pharmacologically
active concentrations. It will be appreciated that
because of this short half-life, repeated injection or
infusion of adenosine may be necessary during cardiac
imaging in accordance with this embodiment of the
invention; by way of example, an initial administration
of 150 μg/kg of adenosine may be made substantially

simultaneously with administration of the contrast agent
composition, followed 10 seconds later by slow injection
of a further 150 μg/kg of adenosine, e.g. over a period
of 20 seconds.

5   Contrast agent preparations in accordance with the
invention may advantageously be employed as delivery
agents for bioactive moieties such as therapeutic drugs
(i.e. agents having a beneficial effect on a specific
disease in a living human or non-human animal),
10   particularly to targeted sites. Thus, for example,
therapeutic compounds may be present in the dispersed
gas or may be linked to part of the stabilising
material, e.g. through covalent or ionic bonds, if
desired through a spacer arm.

15   The controllable growth properties of the dispersed
gas may be utilised to bring about its temporary
retention in the microvasculature of a target region of
interest, and thus retention of the gas and associated
therapeutic compound in a target structure is
20   particularly advantageous.

The therapeutic compound, which may if desired be
coupled to a site-specific vector having affinity for
specific cells, structures or pathological sites, may be
released as a result of, for example, stretching or
25   fracture of the gas-stabilising material caused by
growth of the dispersed gas, solubilisation of the
stabilising material, or disintegration of gas-
microbubbles or gas-containing microparticles. Where a
therapeutic agent is chemically linked to the gas-
30   stabilising material, the linkage or any spacer arm
associated therewith may advantageously contain one or
more labile groups which are cleavable to release the
agent. Representative cleavable groups include amide,
imide, imine, ester, anhydride, acetal, carbamate,
35   carbonate, carbonate ester and disulphide groups which
are biodegradable *in vivo*, e.g. as a result or
hydrolytic and/or enzymatic action.

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Representative and non-limiting examples of drugs useful in accordance with this embodiment of the invention include antineoplastic agents such as vincristine, vinblastine, vindesine, busulfan,
5 chlorambucil, spiroplatin, cisplatin, carboplatin, methotrexate, adriamycin, mitomycin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopurine, mitotane, procarbazine, dactinomycin (antinomycin D), daunorubicin, doxorubicin hydrochloride, taxol,
10 plicamycin, aminoglutethimide, estramustine, flutamide, leuprolide, megestrol acetate, tamoxifen, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase), etoposide, interferon a-2a and 2b, blood products such as hematoporphyrins or derivatives of the
15 foregoing; biological response modifiers such as muramylpeptides; antifungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine, miconazole or amphotericin B; hormones or hormone analogues such as growth hormone, melanocyte stimulating
20 hormone, estradiol, beclomethasone dipropionate, betamethasone, cortisone acetate, dexamethasone, flunisolide, hydrocortisone, methylprednisolone, paramethasone acetate, prednisolone, prednisone, triamcinolone or fludrocortisone acetate; vitamins such
25 as cyanocobalamin or retinoids; enzymes such as alkaline phosphatase or manganese superoxide dismutase; antiallergic agents such as amelexanox; anticoagulation agents such as warfarin, phenprocoumon or heparin; antithrombotic agents; circulatory drugs such as
30 propranolol; metabolic potentiators such as glutathione; antituberculars such as p-aminosalicylic acid, isoniazid, capreomycin sulfate, cyclosexine, ethambutol, ethionamide, pyrazinamide, rifampin or streptomycin sulphate; antivirals such as acyclovir, amantadine,
35 azidothymidine, ribavirin or vidarabine; blood vessel dilating agents such as diltiazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide

dinitrate, nitroglycerin or pentaerythritol
tetranitrate; antibiotics such as dapsone,
chloramphenicol, neomycin, cefaclor, cefadroxil,
cephalexin, cephradine, erythromycin, clindamycin,
lincomycin, amoxicillin, ampicillin, bacampicillin,
carbenicillin, dicloxacillin, cyclacillin,
picloxacillin, hetacillin, methicillin, nafcillin,
penicillin or tetracycline; antiinflammatories such as
diflunisal, ibuprofen, indomethacin, meclefenamate,
mefenamic acid, naproxen, phenylbutazone, piroxicam,
tolmetin, aspirin or salicylates; antiprotozoans such as
chloroquine, metronidazole, quinine or meglumine
antimonate; antirheumatics such as penicillamine;
narcotics such as paregoric; opiates such as codeine,
morphine or opium; cardiac glycosides such as
deslaneside, digitoxin, digoxin, digitalin or digitalis;
neuromuscular blockers such as atracurium mesylate,
gallamine triethiodide, hexafluorenium bromide,
metocurine iodide, pancuronium bromide, succinylcholine
chloride, tubocurarine chloride or vecuronium bromide;
sedatives such as amobarbital, amobarbital sodium,
apropbarbital, butabarbital sodium, chloral hydrate,
ethchlorvynol, ethinamate, flurazepam hydrochloride,
glutethimide, methotrimeprazine hydrochloride,
methyprylon, midazolam hydrochloride, paraldehyde,
pentobarbital, secobarbital sodium, talbutal, temazepam
or triazolam; local anaesthetics such as bupivacaine,
chloroprocaine, etidocaine, lidocaine, mepivacaine,
procaine or tetracaine; general anaesthetics such as
droperidol, etomidate, fentanyl citrate with droperidol,
ketamine hydrochloride, methohexital sodium or
thiopental and pharmaceutically acceptable salts (e.g.
acid addition salts such as the hydrochloride or
hydrobromide or base salts such as sodium, calcium or
magnesium salts) or derivatives (e.g. acetates) thereof;
and radiochemicals, e.g. comprising beta-emitters. Of
particular importance are antithrombotic agents such as

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vitamin K antagonists, heparin and agents with heparin-like activity such as antithrombin III, dalteparin and enoxaparin; blood platelet aggregation inhibitors such as ticlopidine, aspirin, dipyridamole, iloprost and abciximab; and thrombolytic enzymes such as streptokinase and plasminogen activator. Other examples of therapeutics include genetic material such as nucleic acids, RNA, and DNA of natural or synthetic origin, including recombinant RNA and DNA. DNA encoding certain proteins may be used in the treatment of many different types of diseases. For example, tumour necrosis factor or interleukin-2 may be provided to treat advanced cancers; thymidine kinase may be provided to treat ovarian cancer or brain tumors; interleukin-2 may be provided to treat neuroblastoma, malignant melanoma or kidney cancer; and interleukin-4 may be provided to treat cancer.

Contrast agent preparations in accordance with the invention may be used as vehicles for contrast-enhancing moieties for imaging modalities other than ultrasound, for example X-ray, light imaging, magnetic resonance and, more preferably, scintigraphic imaging agents. Controlled growth of the dispersed gas phase may be used to position such agents in areas of interest within the bodies of subjects, which may then be imaged.

Contrast agent preparations in accordance with the invention may also be used as vehicles for therapeutically active substances which do not necessarily require release from the preparation in order to exhibit their therapeutic effect. Such preparations may, for example, incorporate radioactive atoms or ions such as beta-emitters which exhibit a localised radiation-emitting effect following growth of the dispersed gas phase and temporary retention of the agent at a target site. It will be appreciated that such agents should preferably be designed so that subsequent shrinkage and cessation of retention of the

dispersed gas does not occur until the desired
therapeutic radiation dosage has been administered.

Contrast agent preparations in accordance with the
invention may additionally exhibit therapeutic
5   properties in their own right. Thus, for example, the
dispersed gas may be targeted to capillaries leading to
tumours and may act as cell toxic agents by blocking
such capillaries. Concentrations of dispersed gas in
capillaries may also enhance absorption of ultrasonic
10   energy in hyperthermic therapy; this may be used in, for
example, treatment of liver tumours. Irradiation with a
relatively high energy (e.g. 5 W) focused ultrasound
beam, e.g. at 1.5 MHz, may be appropriate in such
applications.
15   The following non-limitative Examples serve to
illustrate the invention.

Preparation 1

Phosphatidylserine-stabilised perfluorobutane
microbubble dispersion

Hydrogenated phosphatidylserine (5 mg/ml in a 1% w/w
solution of propylene glycol in purified water) and
perfluorobutane gas were homogenised in-line at 7800 rpm
and ca. 40°C to yield a creamy-white microbubble
dispersion. The dispersion was fractionated to
substantially remove undersized microbubbles (<2 μm) and
the volume of the dispersion was adjusted to the desired
microbubble concentration by adding aqueous sucrose to
give a sucrose concentration of 92 mg/ml. 2 ml portions
of the resulting dispersion were filled into 10 ml flat-
bottomed vials specially designed for lyophilisation,
and the contents were lyophilised to give a white porous
cake. The lyophilisation chamber was then filled with
perfluorobutane and the vials were sealed. Prior to
use, water was added to a vial and the contents were
gently hand-shaken for several seconds to give a
perfluorobutane microbubble dispersion.

Preparation 2

Phosphatidylserine-stabilised perfluorobutane
microbubble dispersion

Hydrogenated egg phosphatidylserine (50 mg) was
dispersed in water (9.75 ml, reversed osmosis quality)
containing propylene glycol (0.25 ml) by heating to 80°C
for about 2 minutes, followed by cooling to room
temperature. 1 ml portions of the dispersion were
transferred into ten 2 ml chromatography vials. The
headspaces were flushed with perfluorobutane gas and the
vials were capped and shaken on an Espe CapMix® shaker
for 45 seconds, yielding samples of a milky white

microbubble dispersion. The microbubble dispersions were collected in two 7 ml vials which were capped and placed on a roller table for two days. Later the samples were stored standing at room temperature until use.

Preparation 3

Sodium dodecyl sulphate solution

Sodium dodecyl sulphate (2.09 g) was added to water (7 ml, reversed osmosis quality) at room temperature to obtain a clear solution.

Preparation 4

Brij 99 solution

Brij 99 (2.84 g) was added to water (9.5 ml, reversed osmosis quality) and placed on a roller table overnight. A clear, slightly viscous solution was obtained.

Preparation 5

Sodium dodecyl sulphate/n-butanol/water microemulsion

Sodium dodecyl sulphate (12.5810 g), n-butanol (27.0813 g) and water (17.07 g, reversed osmosis quality) were mixed and diluted further with water to a water content of 30% w/w, yielding a clear, transparent microemulsion.

EXAMPLE 1

Microbubble aggregation by calcium chloride

One drop of the microbubble dispersion prepared in Preparation 1 was placed on an object glass for

microscopy investigation. The sample was covered with a
cover glass and placed under a microscope. Droplets of
a 50 mg/ml calcium chloride solution in water were added
to the edge of the cover glass so that the solution
5   penetrated into the microbubble dispersion. The
behaviour of the microbubble dispersion as the calcium
chloride solution front moved was recorded on a video
tape. Microbubble aggregates with larger dimensions
than the initial microbubbles were observed to form,
10   demonstrating that microbubbles with potential for
retention in capillary systems were generated.

EXAMPLE 2

15   <u>Microbubble aggregation by sodium dodecyl sulphate</u>

One drop of the microbubble dispersion prepared in
Preparation 2 was placed on an object glass and covered
by a cover glass. The sample was placed under a Zeiss
20   Axioskop optical microscope equipped with a 10 x
objective and a camera connected to a computer and an
image analysis system. A droplet of the sodium dodecyl
sulphate solution from Preparation 3 was placed on the
edge of the coverglass, so that the solution penetrated
25   under the coverglass and into the microbubble
dispersion. The behaviour of the microbubble dispersion
as the sodium dodecyl sulphate solution front moved was
recorded, showing formation of microbubble aggregates
with larger dimensions than the initial microbubbles,
30   demonstrating the formation of microbubbles with
potential for retention in capillary systems.

EXAMPLE 3

35   <u>Microbubble aggregation by Brij 99</u>

The procedure described in Example 2 was repeated using

the same microbubble dispersion from Preparation 2 but
adding the Brij 99 surfactant solution from Preparation
4 to the edge of the coverglass. The experiment showed
formation of microbubble aggregates with larger

5   dimensions than the initial microbubbles, demonstrating
the formation of microbubbles with potential for
retention in capillary systems.

EXAMPLE 4

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Microbubble growth by ethanol

The procedure described in Example 2 was repeated using
the same microbubble dispersions from Preparation 2 but

15   adding pure ethanol to the edge of the coverglass. The
experiment showed growth of microbubbles resulting in
microbubbles of larger size than the initial
microbubbles, thereby demonstrating the formation of
microbubbles with potential for retention in capillary

20   systems.

EXAMPLE 5

Microbubble growth by isopropanol

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The procedure described in Example 2 was repeated using
the same microbubble dispersion from Preparation 2 but
adding pure isopropanol to the edge of the coverglass.
The experiment showed growth of microbubbles resulting

30   in microbubbles of larger size than the initial
microbubbles, thereby demonstrating the formation of
microbubbles with potential for retention in capillary
systems.

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EXAMPLE 6

Microbubble growth by acetone

The procedure described in Example 2 was repeated using
the same microbubble dispersion from Preparation 2 but
adding pure acetone to the edge of the coverglass. The
experiment showed growth of microbubbles resulting in
microbubbles of larger size than the initial
microbubbles, thereby demonstrating the formation of
microbubbles with potential for retention in capillary
systems.

EXAMPLE 7

Microbubble aggregation and growth by a sodium dodecyl
sulphate/n-butanol/water microemulsion

One droplet of the microbubble dispersion from
Preparation 2, diluted three times with water was placed
on an object glass and covered by a coverglass. The
sample was placed under a Zeiss Axioskop optical
microscope equipped with a 40 x objective and a camera
connected to a computer and an image analysis system. A
droplet of the sodium dodecyl sulphate/n-butanol/water
microemulsion from Preparation 5 was placed on the edge
of the coverglass, so that the microemulsion penetrated
under the coverglass and into the microbubble
dispersion. The behaviour of the microbubble dispersion
as the microemulsion front advanced was recorded,
showing formation of microbubble aggregates with larger
dimensions than the initial microbubbles. A short time
afterwards, the microbubbles in the aggregates fused or
coalesced to give larger microbubbles, demonstrating the
formation of microbubbles with potential for retention
in capillary systems.